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The cytoskeletal protein vinculin is acylated by myristic acid

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In non-muscle cells the mechanism by which microfilament bundles interact with the plasma membrane is unclear. Vinculin, a 130 kDa protein found in adhesion plaques, has been postulated to have a role as a membrane anchor for microfilaments and we have investigated the biochemistry of this molecule in more detail. We report that a fraction of vinculin in chick embryo fibroblasts is acylated by myristic acid. This modification was present in both membrane-bound, cytoskeletal and cytosolic vinculin and thus did not determine preferential subcellular localisation. Myristic acid was also present in vinculin from cells transformed by Rous sarcoma virus.

Cytoskeleton; Vinculin; Acylation; Myristic acid

1. INTRODUCTION

In recent years much attention has focussed on the interaction of the actin-containing microfilament system with the plasma membrane, and while the membrane cytoskeleton in erythrocytes is now well documented, the situation is less clear in other cells whose cytoskeletal apparatus appears to be in a more dynamic state. One of the candidates for a protein which anchors microfilaments to the membrane is vinculin, a 130 kDa protein present in the adhesion plaques of many cells and at cell-cell junctions [1–4] and it has been proposed that tyrosine-specific phosphorylation of vinculin in RSV-transformed cells leads to a disruption of this function, thus resulting in the rounded morphology characteristic of cells transformed by RSV [5,6]. The paucity of biochemical information about vinculin has led us to examine in more detail biochemical and functional aspects of this molecule in an attempt to gain an insight into the mechanism by which vinculin might interact with the membrane cytoskeleton.

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Abbreviations: CEF, chick embryo fibroblasts; RSV, Rous sarcoma virus; SDS-PAGE, SDS-polyacrylamide electrophoresis; RIPA, radioimmune precipitation; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; DMEM, Dulbecco's minimal essential medium; CSK, cytoskeleton

2. MATERIALS AND METHODS

2.1. *Cells*

Chick embryo fibroblasts were grown in DMEM supplemented with 10% tryptose phosphate broth, 5% newborn calf serum and 1% chick serum, and transformed by Prague C strain RSV as described [7].

2.2. *Radiolabelling and immune precipitation*

CEF were labelled for 3 h with 50 μCi/ml [^{35}S]methionine or 10 μCi/ml [^{14}C]myristate (Amersham) in serum-free DMEM containing 5 mM sodium pyruvate. After labelling the cells were washed and either directly lysed in SDS sam-

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ple buffer, or lysed in RIPA buffer and immune precipitated using a rabbit anti-vinculin serum [8], followed by separation by SDS-PAGE. The gels were then prepared for fluorography using EN^{3}HANCE (Amersham) as described by the manufacturer.

2.3. *Cell fractionation*

A crude membrane fraction was obtained by hypotonic swelling followed by dounce homogenisation. After low-speed centrifugation to remove nuclei the homogenates were centrifuged at 30000 × *g* for 30 min to obtain a crude membrane fraction (pellet) and cytosolic fraction (supernatant) [7]. Cytoskeletons were prepared by the method of Ben-Ze'ev et al. [9]. Labelled cells were extracted in CSK buffer (10 mM Mes, pH 6.8, 100 mM KCl, 300 mM sucrose, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1% NP40, 2 mM PMSF) for 5 min, washed twice in CSK buffer and the insoluble 'cytoskeleton' dissolved in RIPA buffer. An equal volume of double-strength RIPA buffer was added to the soluble fraction and these were then subjected to immune precipitation.

2.4. *Hydroxylamine treatment*

The gel was placed in 1 M hydroxylamine (pH 8.5) or 0.1 M Tris (pH 8.5) for 2 h at room temperature. After washing, the gels were re-fixed and prepared for fluorography.

2.5. *Lipid analysis*

The [^{14}C]myristate-labelled vinculin band was excised and hydrolysed with 6 N HCl under nitrogen for 18 h at 110°C. The hydrolysate was extracted twice with benzene, the organic phase dried under N_2 and the fatty acids separated by thin-layer chromatography on KC18 reversed-phase plates in acetic acid/acetonitrile [10]. 0.5 cm sections were scraped into Aquasol and counted. Authentic palmitic acid and myristic acid were used as standards.

3. RESULTS

When CEF were labelled with [^{14}C]myristic acid and the resultant proteins separated by SDS-PAGE, a series of labelled bands distinct from that seen with [^{35}S]methionine-labelled cells were apparent, with molecular masses ranging from 30 to 180 kDa (fig.1). We were particularly interested in the myristylated band of 130 kDa, since previous work has shown that vinculin has this apparent molecular mass. The identity of this band with vinculin was confirmed by immune precipitation of vinculin from [^{14}C]myristate- or [^{35}S]methionine-labelled CEF. SDS-PAGE revealed [^{14}C]myristate-labelled band specifically precipitated by a rabbit anti-vinculin antiserum which co-migrated with ^{35}S-labelled vinculin (fig.1). The specificity of the lipid linked to vinculin was examined by comparing vinculin precipitated from cells labelled with either [^{14}C]myristate or [^{14}C]palmitate (fig.2). Quantitation revealed that vinculin from palmitate-labelled CEF contained only 23% of the counts of vinculin from myristate-labelled CEF. This [^{14}C]myristate label was resistant to treatment with hydroxylamine (fig.2). To ascertain that the label was lipid, [^{14}C]myristate-labelled vinculin was excised from the gel after SDS-PAGE and the lipids released by acid hydrolysis were analysed. The major labelled lipid present co-migrated with authentic myristic acid, however some palmitic acid was detected (fig.3), and also a peak representing some unknown lipid which migrated more slowly than either myristate or palmitate. From scintillation counting of [^{14}C]myristate-labelled vinculin and protein determination of stained gels it was estimated that the stoichiometry of lipid/protein was 0.04, suggesting only 4% of vinculin molecules in the cell contain lipid. However,

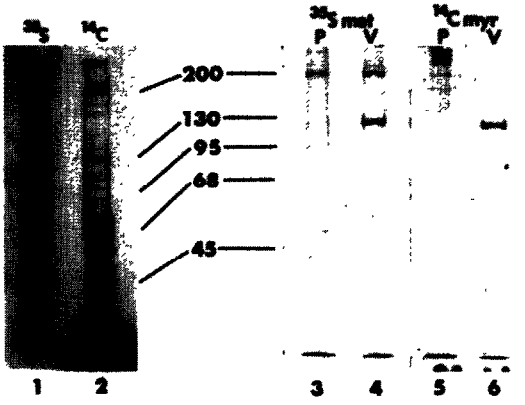

Fig.1. Immune precipitation of vinculin from [^{35}S]methionine- or [^{14}C]myristate-labelled CEF. Lanes: 1,2, total protein; 3–6, immune precipitation; P, pre-immune serum; V, anti-vinculin serum.

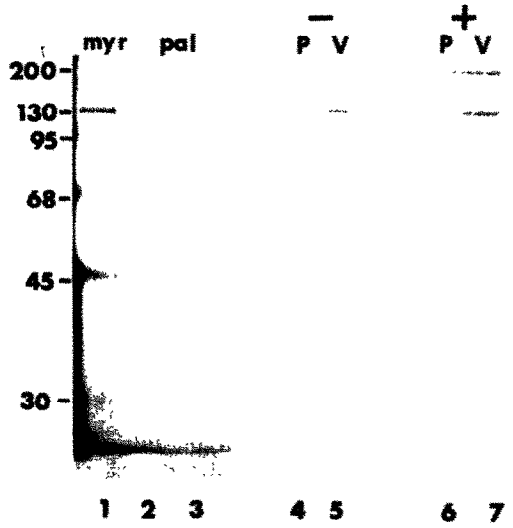

Fig.2. Comparison of myristate and palmitate labelling of vinculin and stability to hydroxylamine. Lanes: 1, anti-vinculin, [^{14}C]myristate; 2, pre-immune, [^{14}C]myristate; 3, [^{14}C]palmitate anti-vinculin; 4, [^{14}C]myristate pre-immune, buffer treated; 5, [^{14}C]myristate anti-vinculin, buffer treated; 6, [^{14}C]myristate, pre-immune hydroxylamine treated; 7, [^{14}C]myristate anti-vinculin, hydroxylamine treated.

this may be an underestimate since over a 3 h labelling period it is unlikely that the protein will be labelled to equilibrium. We were interested in whether myristate-containing vinculin showed a preferential localisation to any particular cellular fraction. When CEF were fractionated into crude membrane and cytosolic fractions, the proportion

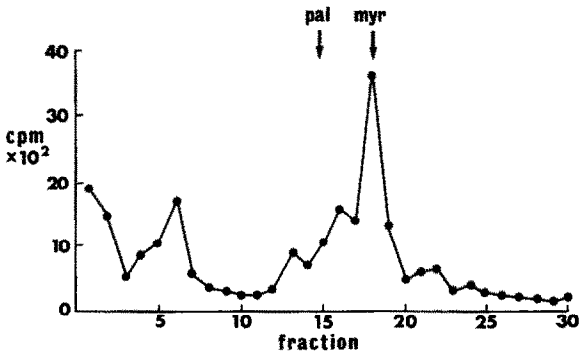

Fig.3. Identification of fatty acids liberated after hydrolysis of [^{14}C]myristate-labelled vinculin.

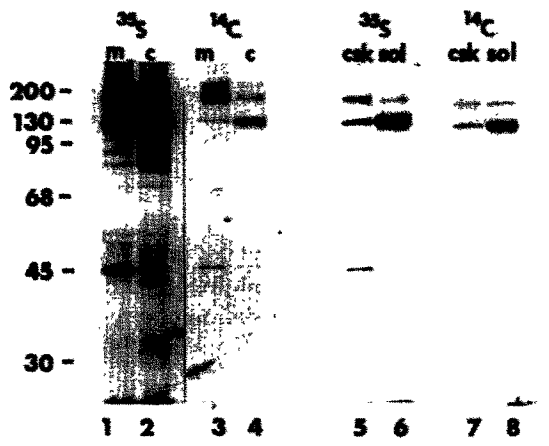

Fig.4. Immune precipitation of [^{35}S]methionine- or [^{14}C]myristate-labelled vinculin from crude membrane and cytosolic fractions (lanes 1–4) or cytoskeletal and soluble fractions (lanes 5–8); m, crude membranes; c, cytosolic; csk, cytoskeleton; sol, detergent-soluble.

of [^{14}C]myristate-labelled vinculin in each compartment closely resembled that of [^{35}S]methionine-labelled vinculin, with approx. 10% of the vinculin in the crude membrane fraction and 90% in the cytosol (fig.4). Labelled CEF were also extracted into a cytoskeletal-associated fraction and a detergent-soluble fraction by published procedures [9,11]. The proportions of [^{14}C]myristate-labelled vinculin in each fraction were also very similar to that of [^{35}S]methionine-labelled vinculin, with about 90% freely soluble and 10% cytoskeletal (fig.4); these proportions are similar to that of vinculin in human platelets [12]. Thus, myristylation of vinculin does not determine preferential localisation to a membrane or cytoskeletal fraction.

Since the localisation of vinculin is drastically affected by transformation, we determined the extent of myristylation of vinculin in RSV-transformed CEF to examine whether there was any change in the amount of lipid associated. RSV-transformed CEF contained about 60–70% of the vinculin of untransformed cells as ascertained by [^{35}S]methionine labelling, however there was a comparable reduction in the amount of [^{14}C]myristate-labelled vinculin in RSV-transformed cells, thus the stoichiometry of lipid to protein remained constant (fig.5).

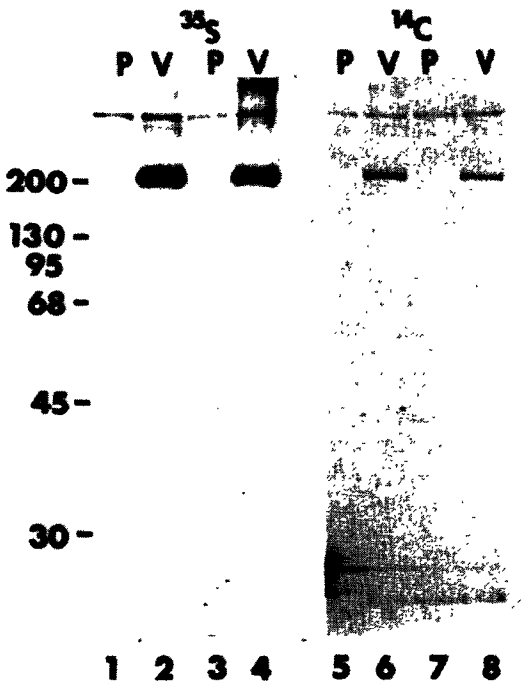

Fig.5. Vinculin from untransformed (lanes 1,2,5,6) and RSV-transformed (lanes 3,4,7,8) CEF. Cells were labelled with [^{35}S]methionine (lanes 1–4) or [^{14}C]myristate (lanes 5–8). P, pre-immune serum; V, anti-vinculin.

4. DISCUSSION

A function for the cytoskeletal protein vinculin has yet to be elucidated, and a study of the biochemical properties of this protein may provide an insight into this problem. Our findings that a proportion of vinculin is myristylated suggest that this molecule has the potential to interact with membranes, although our fractionation experiments do not support this. It may be that this interaction is not stable under the conditions employed, and that other factors are important. Myristylation is necessary for the attachment of pp60^{v-src} to the plasma membrane since mutant proteins lacking myristate are largely cytoplasmic [13], although additional factors may also be involved in this localisation [17]. Calcineurin B [14] and the cAMP-dependent protein kinase catalytic subunit [15] contain myristic acid yet are cytoplasmic, so it is clear that myristylation per se is not sufficient for membrane localisation. Some membrane-associated components such as p21 of Harvey murine sarcoma virus and transferrin receptor have been shown to contain another lipid modification, addition of palmitic acid [16,17], and it is of interest to note that in erythrocytes the membrane-cytoskeletal component ankyrin has been reported to contain palmitic acid [18]. The attachment of myristic acid to other proteins has been shown to be mediated via an amide bond formed between N-terminal glycine and myristate [13] and it may be that a similar bond is involved here since the bond is stable to hydroxylamine treatment, although the amino acid sequence of vinculin has not yet been determined. Consistent with this is the finding that the amino-terminus of vinculin is blocked when amino acid sequencing of the protein is attempted (Critchley, D.R., personal communication).

Apart from its cellular localisation there is no functional evidence to support a membrane anchor role for vinculin, since vinculin does not act as an actin-binding protein [19,20], and has not been found in purified plasma membranes of HeLa cells [20]. However, under appropriate conditions vinculin can associate with plasma membranes independently of actin [21,22] and Burridge and co-workers have described the interaction of vinculin with another protein, talin [23], which in turn may interact with the cell membrane receptor for fibronectin [24]. Furthermore, binding experiments and crosslinking-labelling experiments have shown that vinculin will incorporate into synthetic lipid bilayers containing acidic phospholipids and that incorporation of a photoactivatable ^{3}H-labelled lipid component under these conditions results in the labelling of vinculin [25,26]. Thus, given the proper conditions vinculin might interact with the plasma membranes of cells and tightly associate with lipid domains in the membrane. The finding that PDGF-stimulated reorganisation of vinculin in epithelial cells is abrogated by inhibitors of phospholipase would also suggest that a lipid moiety associated with vinculin might be functionally important [27]. Myristylation may provide a means to mediate this phenomenon.

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